BETTY M. WATTS, JEAN KENDRICK, MARELYNN W. ZIPSER, BOBBIE HUTCHINS, and BASSIMA SALEH

Department of Food and Nutrition, Florida State University, Tallahassee, Florida 32306

Enzymatic Reducing Pathways in Meat

SUMMARY

Methods for measuring reducing capacity of meats are described. These include changes in oxidation-reduction potentials of ground meat and changes in oxygen tension of meat slurries, as well as reduction of metmyoglobin. Except for a small residual utilization of oxygen in meat slurries (ascribed to nonenzymatic oxidation), all reductive activity in meat can be stopped by inhibitors of DPNH oxidation via the electron transport chain. Added DPN accelerates all reductive activity. Metmyoglobin reduction does not occur until oxygen has substantially disappeared from the meat.

Meat contains little or no succinate. Added succinate greatly accelerates oxygen utilization, but affects metmyoglobin reduction only indirectly by establishing anaerobic conditions more rapidly. It is concluded that both oxygen utilization and metmyoglobin reduction in meat are normally mediated through DPN.

INTRODUCTION

The color of fresh meat is largely determined by the relative proportions and distribution of the three meat pigments, purple reduced myoglobin (M), red oxymyoglobin (MO_2), and brown metmyoglobin (M^+). The last pigment is particularly undesirable, not only from the color standpoint, but also because ferric hemes act as catalysts in the oxidation of unsaturated lipids.

The proportion of M to MO_2 is influenced by the activity of enzyme systems within the meat. That meat is capable of utilizing oxygen is evident from the simple observation that in oxygen-impermeable wrappings, surface MO_2 quickly dissociates to M. Since the oxygen tension for half saturation of M is 1 to 1.4 mm Hg (George and Stratmann, 1952), this color change obviously denotes a rapid utilization by the meat of the oxygen within the package.

M may also be oxidized to M^+ rather than oxygenated to MO_2. The rate of autoxidation is dependent on oxygen tension and is highest at half saturation (George and Stratmann, 1952). However, meat enzymes can reduce M^+ as well as oxygen (Stewart *et al.*, 1965b). The accumulation of M^+ in stored meats is the resultant of these opposing factors (autoxidation and enzymatic reduction). The purpose of this research is to explore enzymatic pathways by which meat is able to reduce both oxygen and M^+, and the relation between oxygen and M^+ reduction.

A review of the biochemistry of respiratory activity in the living muscle cells and the changes known to occur after slaughter (Bendall, 1962; Lawrie, 1962) leads to some predictions concerning probable reductive pathways in meat. In the living tissue, hydrogen, derived from the reactions of the Krebs cycle, passes to the electron-transport chain (ETC) either by way of DPN and its associated flavoprotein dehydrogenase (FD) or from succinate and the flavoprotein succinic dehydrogenase (FS) (Green and Fleischer, 1962; Hatifi, 1963).

Upon slaughter, oxygen is cut off from the tissues, and a rapid anaerobic glycolysis ensues. In postrigor meat most of the glycogen has been converted to lactic acid, leaving a large pool of lactate. ATP and other high-energy phosphates have disappeared.

On the other hand, many enzymes, including lactic dehydrogenase (LD) and all components of the glycolytic pathway, succinic dehydrogenase and all components of the ETC, remain potentially active in meats even after extended refrigerator storage (Andrews *et al.*, 1952; Bodwell *et al.*, 1965).

If oxygen becomes available again, as when meat is ground or cut surfaces are exposed, the resumption of enzymic oxidase activity would be expected provided suitable hydrogen donors are present. Any succinate present should be rapidly oxidized by way of mitochondrial succinic dehydrogenase and the ETC. This should result in a utilization of oxygen, but there is no known pathway for transfer of electrons from succinate to M^+.

On the other hand, reduction of DPN^+ to DPNH could lead to reduction of both M^+ and O_2. There is an extensive literature, not reviewed here, on the reduction of ferric hemes by DPNH in artificial systems, red

cell preparations, etc., provided suitable intermediates are present. Although the pyridine nucleotides cannot pass undamaged mitochondrial membranes, there is good evidence that DPNH, generated externally in the cytoplasm, can be oxidized by way of the mitochondrial ETC (Margreth and Azzone, 1964; Cunningham, 1964).

While there are a number of enzyme-substrate systems capable of reducing DPN in living muscle, the activity of these systems in postrigor meat is unexplored. In most cases substrates would be lacking, even if the enzymes are present. As mentioned above, lactate is present in large amounts. Lactate may be oxidized to pyruvate by the DPN-linked enzyme LD:

$$CH_3CHOHCOOH + DPN^+ \rightleftarrows CH_3COCOOH + DPNH + H^+$$

The equilibrium of this reaction lies far to the left, so that lactate oxidation would require effective removal of the pyruvate and DPNH.

With the above background in mind, the reductive pathways shown in Fig. 1 may be advanced as a hypothetical scheme for the reduction of oxygen and metmyoglobin in meat.

To test this hypothetical scheme, we have tried the effect of added DPN and succinate and of known inhibitors on reducing activities in meat. Four inhibitors have proven useful in this study. Oxalate acts as a competitive inhibitor for lactate in the LD region (Ottolenghi and Denstedt, 1958; Novoa *et al.*, 1959), thus blocking the overall reaction in position 1, provided lactate is the substrate. Ernster and Lee (1964) have reviewed the literature on the other three inhibitors. Amytal and rotenone both block reduction of DPNH in the flavoprotein region, position 2, but have no effect on succinate oxidation. Antimycin A blocks electron transport from DPNH or succinate at position 3. Unfortunately, inhibitors in the cytochrome A region are compounds such as cyanide, azide, etc., which combine with heme iron and therefore react with M or M^+ as well as with cytochrome A. Since they interfere with determination of M^+ reduction, they were not used in this work.

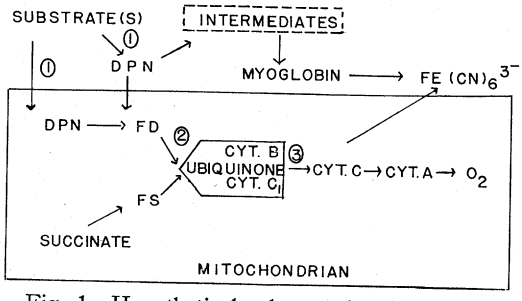

Fig. 1. Hypothetical scheme of reductive pathways in meat. Arrows indicate direction of electron flow. Electron transport is blocked at position 1 by oxalate (if the substrate is lactic acid), at position 2 by amytal and rotenone, and at position 3 by antimycin A.

METHODS

Preparation of meat. Meat was obtained from local packing houses. Both pork hams and beef (eye of round) were used. The preslaughter history of the animals was not known. All meat was trimmed of external fat and ground twice just before each experiment. The ground meat for any one experiment was mixed thoroughly, and weighed portions were treated with additives as described under each experiment. Three kinds of tests, described below, were used to measure the reductive capacity of the controls and treated samples.

M^+-reducing activity was measured as described by Stewart *et al.* (1965b). The method consists of first oxidizing all of the M to M^+ in ground meat by the addition of a slight excess of $K_3Fe(CN)_6$ and then following the reduction of the M^+ during a 15-to-90-min period by reflectance spectrophotometry (Stewart *et al.*, 1965a).

The addition of any oxidizing agent has obvious drawbacks in a study of enzymatic reducing activity. The oxidizing agent may itself be reduced enzymatically. Ferricyanide is known to be reduced by electrons, which may come off the ETC at various points, but mainly at the cytochrome *c* level (Eastbrook, 1961). Magos (1964) investigated a number of methemoglobin-forming agents and concluded that nitrite was preferable to ferricyanide because it did not oxidize sulfhydryl groups in the protein or cause denaturation. However, nitrite may be reduced, at least under anaerobic conditions, by reduced cytochrome *c* and cytochrome oxidase (Walters and Taylor, 1965).

In view of these uncertainties, and of its use in meat curing, nitrite was used as the metmyoglobin-forming agent in some of the experimental work. Preliminary work established that .006% NO_2^- was sufficient to oxidize completely the M in most samples of meat. Oxidation was less rapid by nitrite than by ferricyanide; the meat had to be stirred longer to obtain complete oxidation. The standard procedure was to add 1 ml of freshly pre-

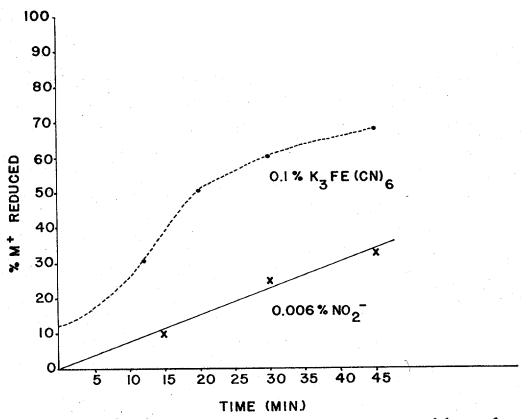

Fig. 2. $K_3Fe(CN)_6$ vs. NO_2^- as an oxidant for studying M^+-reducing activity of meat.

pared $NaNO_2$ solution (0.3%NO_2^-) to 50 g meat, stir for 12½ min, and then begin readings on the Spectronic 505 at 15 min (designated as zero time in the reduction data). The enzymatic reduction of M^+ occurred more slowly when NO_2^- was used as the oxidizing agent than when ferricyanide was used, and reduction with NO_2^- was generally linear with time, whereas sigmoid curves with variable lag periods were obtained with ferricyanide (Fig. 2).

The pigment obtained upon reduction of M^+ in the presence of NO_2^- was nitric oxide myoglobin. This pigment, like reduced myoglobin, was isosbestic with M^+ at 525 mμ, but the wavelength of greatest difference between the nitric oxide pigment and M^+ was at 550–553 mμ. The M^+ determination was therefore modified from the procedure described by Stewart *et al.* (1965a) by substituting the ratio $\frac{K/S\ 550\text{-}553}{K/\ 525}$ for the ratio $\frac{K/S\ 572}{K/S\ 525}$, used to determine M^+ in mixtures of M and M^+. The average ratio was 0.88 for completely oxidized samples, and 1.37 for completely reduced samples. A linear relation was assumed between these limiting values and the percent of the total pigment present as M^+.

Oxidation-reduction potentials. In some of the earlier experiments, before suitable methods for measuring oxygen tension had been developed, changes in oxidation-reduction potential of ground meat were used as supplementary evidence for reducing capacity of the meat. The ground meat was brought to room temperature as in the spectrophotometric analysis and stirred for 3½ min. Test substances were then added and mixed 3½ min. The meat was packed tightly into a 50-ml beaker, electrodes were inserted, and readings were begun in another 1½ min (designated zero time).

The Beckman zeromatic pH meter, model 9600, was used for the measurements, A platinum electrode, No. 39276 was used in combination with the calomel reference electrode, No. 39170. Readings were usually taken at intervals over a period of 30 min.

The pattern of potential change was different for each lot of ground meat, but the pattern was reproducible when different portions of the same lot of meat were similarly treated. Also when the same sample of meat was removed from the beaker and restirred with air, the potential returned to its original high value and a second potential curve usually duplicated the first.

The drop in potential does not, of course, give specific information either on oxygen utilization or M^+ reduction; it is affected by the concentration of many metabolite pairs and their associated enzymes (Wurmser and Banerjee, 1964). However, under the conditions of these experiments, where the initial values are under aerobic conditions, and the packed meat utilizes the oxygen present, rate of fall in oxidation-reduction potentials of different samples of meat seemed to correlate reasonably well with enzymatic reduction by way of the ETC. The fall in potential could be completely blocked by ETC inhibitors.

Oxygen consumption. Changes in oxygen tension were measured with a polarographic oxygen analyzer, Beckman model 777. In order to obtain meaningful measurements with this instrument it is necessary to work with meat slurries which can be adequately stirred during the test period. The following technique was found to yield slurries which generally maintained a linear rate of oxygen consumption for at least a 10-min period.

A 50-g portion of ground meat plus any additives under investigation and sufficient 0.25*M* sucrose solution to give a total liquid volume of 100 ml is homogenized in a 500-ml Virtis flask for 2 min at a rheostat setting of 40. The meat and all solutions are cold, and the blending is done in an ice bath. The cold homogenate is run through a large strainer or a double layer of cheesecloth to remove strands of connective tissue, and brought quickly to 25°C. A 50-ml Erlenmeyer flask with Teflon-covered magnet is filled with the homogenate and placed on an asbestos-covered foam pad on a magnetic stirrer. The sensor of the oxygen analyzer is then placed in the flask (it fits snugly in the neck), and the recording is begun. The entire preparation time, up to the beginning of the recording, is 8 min. Deviations in preparation time, failure to keep the homogenate cold during blending, or omission of the sucrose (used to protect cell structures from osmotic damage) result in poor reproducibility and decreases in the rate of oxygen consumption during the test period.

Usually the slope of the change in oxygen tension (PO_2) was measured from 2 to 5 min. At 5 min a test substance would be added to the flask and the new slope measured from 7 to 8 min. All slopes are expressed as drop in oxygen tension in mm Hg per minute. Changes in oxygen tension could not be translated precisely into moles of oxygen consumed, since the solubility of oxygen in the slurries at any particular oxygen tension is not known. However, accepting the figure of oxygen solubility of 8.4 ppm O_2 in pure water in equilibrium with water-saturated air at 25°C and 760 mm Hg (160 mm Hg PO_2) and correcting this for the decreased solubility of oxygen in the sugar solutions used (92% of the water value) gives a value of 7.7 ppm O_2 at 160 mm Hg PO_2. A fall of 1 mm in the oxygen tension measured is thus very roughly equivalent to a loss of .048 ppm O_2 or to 1.5×10^{-3} μmoles of O_2 per g of slurry.

Addition of inhibitors. Tentative concentrations of each inhibitor necessary to give maximum inhibition were obtained from several sources in the literature and are shown in the results. Except with potassium oxalate, concentrations of each inhibitor higher than those shown were also tried in samples of meat where 100% inhibition had not been obtained. Doubling the concentration gave no additional inhibition. The oxalate was added in phosphate buffer of the same pH as the meat sample, and the same amount of buffer was used in the control. The amount of amytal needed for 50 g meat was dissolved in 1 ml propylene glycol, and rotenone and antimycin A were dissolved in 1 ml ethanol, before addition to the meat. Since both propylene glycol and ethanol increased the reductive capacity of the meat, it was necessary to use controls having the same amount of solvent.

With all of the poisons used, the inhibition disappeared after some time (usually 15 to 50 min). This is apparently due to a loss of the inhibitor itself rather than to the establishment of alternative reductive pathways, since addition of further inhibitor when reduction had begun resulted, again, in complete inhibition.

Anaerobic experiments. To test the ability of the meat to reduce M^+ in the absence of oxygen, two different experimental techniques were employed:

Procedure for ground meat. Fifty-gram portions of the ground meat were placed in Saran bags (6½ × 11¾ inches). A glass cup (1¾ × 1⅛ inches) with lid was also placed in the bag. Nitrogen inlet and outlet tubes and the sensor of the oxygen analyzer were inserted through the opening in the bag and held in place with rubber bands and a clamp.

One minute of nitrogen flushing was sufficient to bring the oxygen tension practically to zero. The meat was mixed for a 3-min period, by external manipulation of the bag, to remove entrapped oxygen from the meat. The oxidizing agents and test substances were injected into the meat mass through the Saran bag. Mixing of meat and additives, under nitrogen, was continued for another 3 min. Finally, while still in the bag and under nitrogen, the meat was packed into the glass cup and covered. After removal from the bag, the cup was sealed with masking tape around the sides and the meat pigments analyzed by spectrophotometry as previously described. The first curve was obtained 4½ min after addition of the oxidizing agent.

Procedure for meat slurries. Slurries were prepared as previously described. After blending, the oxidant, ferricyanide or nitrite, was added. After filtering through cheesecloth the slurry was brought to 25°C (1–1½ min) and transferred to a special cell (see Fig. 3) designed to allow simultaneous measurements of PO_2 and M^+ reduction while flushing with N_2. A Teflon-covered stirring magnet 2½ cm long was placed in the flask, and the calibrated oxygen sensor was inserted and secured with Parafilm. The flask was placed on a magnetic stirrer covered with asbestos screen and a petri-dish top to catch the overflow of slurry. Slurry was added through arm B to fill the cell completely, and the arm was sealed off with Parafilm. The magnetic stirrer was set at a speed of 7, and the recorder of the analyzer was started. The total preparation time from beginning of blending to the time the recorder was

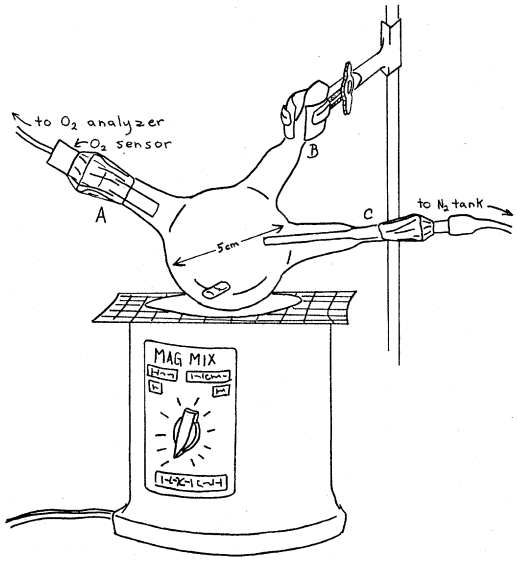

Fig. 3. Specially designed cell for simultaneous measurements of oxygen tension and M^+ reduction in meat slurries.

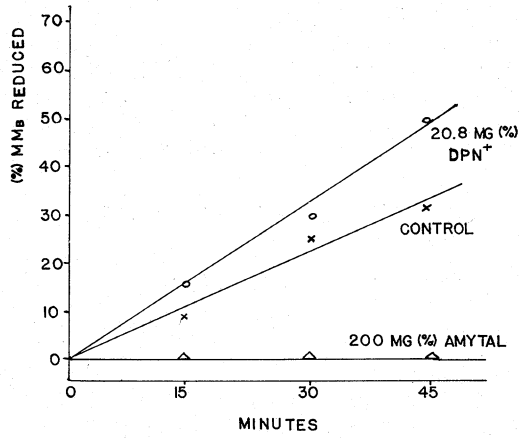

Fig. 4. Effect of DPN and amytal on reduction of M^+ in ground pork. Pigment was first oxidized with nitrite.

turned on was 10 min. The spectral analysis was carried out as with the ground meat sample.

RESULTS

Role of DPN. Evidence for the important role of DPN in the reductive activity of meat was obtained both from the use of inhibitors and also from the addition of DPN. Figs. 4, 5, and 6 show data obtained on the same sample of meat with the three tests described. In this experiment, nitrite was used as the oxidizing agent for the pigment studies, but no oxidizing agent was used in the potential or oxygen tension measurements. It will be seen that amytal blocked completely both M^+ reduction and also the drop in the oxidation-reduction potential. Oxygen consumption in the meat

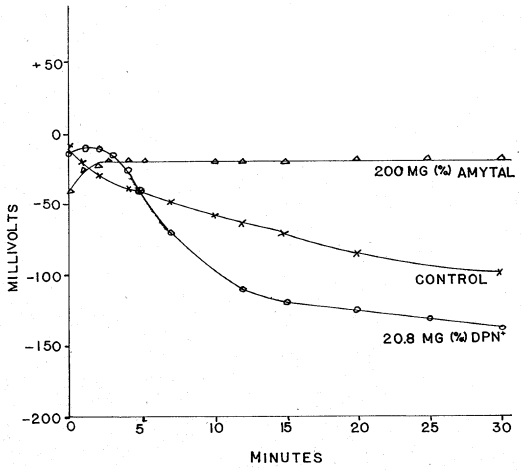

Fig. 5. Effect of DPN and amytal on oxidation-reduction potentials of ground pork.

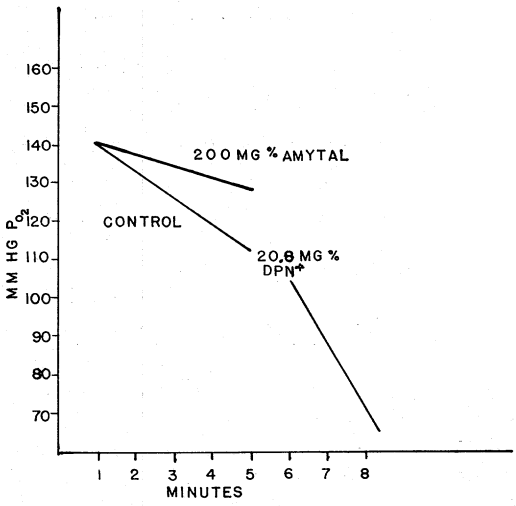

Fig. 6. Effect of DPN and amytal on oxygen utilization in a ground pork slurry.

slurries was inhibited only partially. It was found in all inhibitor experiments with the oxygen analyzer that a residual oxidation of about 2 to 5 mm Hg PO_2 per minute remained even when, as in this experiment, all pigment oxidation and potential change had been stopped in the ground meat. It is probable that this activity represents other oxidations stimulated by the homogenization. This could include nonenzymatic reactions such as heme-catalyzed lipid oxidations. In fact, oxygen consumption of this order of magnitude is observed in slurries from cooked beef.

Table 1 shows the results of five additional experiments with amytal, and one with retenone. Inhibition is generally quite high, approaching 100% with most samples of meat. In Expt. 2, where the inhibition was somewhat less, no more inhibition was obtained even with five times the amount of amytal shown. It is believed that traces of succinate account for the residual oxidation. Malonate, a specific inhibitor for succinate oxidation, was not used in this sample of meat, but was found to give slight, variable inhibition with several samples of meat.

The addition of DPN to meat resulted in increased reductive activity. This is shown in the typical experiment illustrated in Figs. 4, 5, and 6. In eight additional experiments, rate of M^+ reduction was increased 23 to 51% over that of the control. The DPN present in meat at slaughter is progressively

Table 1. Effect of ETC poisons on reducing activity of pork.

Expt. no.	Inhibitor added	M+ reduction: Oxidizing agent	Time of reduction (min)	Control % M+ reduced	Inhibition (%)	Potential changes: Time (min)	Control drop (mv)	Inhibition (%)
1	Amytal, 200 mg%	Ferri	30	66	100	30	91	100
2	Amytal, 200 mg%	Ferri	20	78	84	20	105	29
3	Amytal, 200 mg%	Ferri	20	43	96	20	116	88
	Rotenone, 6 mg%	Ferri	20	43	100	20	116	85
4	Amytal, 200 mg%	NO_2^-	40	29	100	....	....	....
5	Amytal, 200 mg%	NO_2^-	40	31	94	30	90	100
6	Antimycin A, 6 mg%	Ferri	10	7	86	10	65	92
			20	32	90	20	110	70

destroyed by the action of several enzymes which are brought into contact with the nucleotide upon maceration of the tissue. These enzyme systems have been reviewed by Severin *et al.* (1963). The loss of reducing activity when ground meat is refrigerated, noted by Stewart *et al.* (1965b), may be ascribed to loss of DPN. Most of the original reducing activity can be restored by the addition of DPN at the end of the storage period.

The identity of the substrate or substrates (Fig. 1) which supply hydrogen to DPN^+ is not clear at this time. The addition of potassium oxalate, competitive inhibitor for lactate in the LD reaction, gave variable results with different samples of meat. At an oxalate level of 0.5 g per 100 g meat, the general pattern seemed to be an initial inhibition of reductive activity, by all three tests, for the first 10–20 min. This was followed by a marked acceleration, so that at 45 min the oxalate-treated samples showed greater M^+ reduction than the controls. Larger concentrations, up to 1%, generally increased the time of inhibition, although this varied with the sample of meat. Further work on the addition of DPN-linked substrates to meat will be reported later.

Intermediates between DPNH and M^+ are also unknown at this time. DT diaphorase (also known as menadione reductase) is a cytoplasmic flavoenzyme which catalyzes the reduction of various quinones, which in turn reduce ferric heme compounds (Ernster *et al.* 1962; Conover and Ernster, 1962). However, dicoumarol, a specific inhibitor for this enzyme, did not inhibit M^+ reduction in meat at a concentration of 6 mg%.

Relation between M^+ reduction and oxygen utilization. The fact that inhibitors of the ETC also inhibit M^+ reduction does not mean that M^+ is being reduced by way of the ETC. Rather, it indicates that oxygen must be substantially eliminated before M^+ reduction begins. This is indicated by the fact that all spectrophotometric curves obtained during the reduction of M^+ show only mixtures of M^+ and M, without any MO_2, where ferricyanide was used as the oxidant. Apparently, hydrogen or electrons from DPNH react preferentially with oxygen as long as any oxygen remains.

This is shown in Table 2, containing results of an experiment in which simultaneous

Table 2. Effect of oxygen tension and DPN on reduction of metmyoglobin in beef slurries.

Sample	Time (min)	PO_2 (mm Hg)	% M+ reduction
Control [a]	0	54.4	0
	10	32.0	0
	20	0	10
	30	0	26
DPN [b]	0	65.6	....
	10	9.6	0
	20	0	35
	30	0	56
Control $+N_2$ [c]	0	....	0
	10	0	1
	20	0	14
	30	0	25
DPN $+N_2$ [c]	0	....	....
	10	0	26
	20	0	46
	30	0	73

[a] Control contained: 200 mg% nicotinamide, 3 mg% CTC and 30 mg% $K_3Fe(CN)_6$.
[b] DPN contained: Same as control + 40 mg% DPN.
[c] N_2 bubbled through at zero time.

O_2 tension and M^+ reduction measurements were carried out on meat slurries, with and without added DPN. For this experiment, the special flask described earlier (Fig. 2) was used.

The addition of DPN to ground meat oxidized with ferricyanide under anaerobic conditions (Table 3) usually resulted in a very rapid reduction of M^+, with no lag period. In fact, much reduction takes place during the mixing period, before the first spectrophotometric curve is obtained. On the other hand, when meat is not deaerated before the addition of DPN, there is normally a lag period before M^+ reduction begins. Oxygen and any excess ferricyanide or nitrite are presumably being reduced during the lag period. Fig. 7 shows a typical set of data on meat containing DPN under anaerobic versus aerobic conditions.

Oxygen utilization is much more rapid in the presence of succinate. Most of the samples of meat tested contained no more than a trace of succinate, as shown by the fact that malonate gave very slight inhibition of reducing activity. The addition of 100–200 mg succinate per 100 g meat increased the rate of oxygen utilization in meat slurries to 3–9 times that of the control. Thus, although there is no direct pathway from succinate to M^+, the rapid establishment of anaerobic conditions in succinate-treated samples effectively shortened or eliminated the lag period in M^+ reduction.

Table 3. The effect of DPN on metmyoglobin reduction in ground beef under anaerobic conditions.

Experiment	Time (min)	Metmyoglobin (% of total pigment) Sample: Control	Sample: (+ DPN, 40 mg%)
A	0	100	86
	10	99	51
	20	86	22
	30	75	8
	40	65	0
B	0	100	52
	10	95	10
	20	82	6
	30	68	0
C	0	99	100
	10	99	94
	20	96	68
	30	92	36
	40	86	14
	50	77	5
	60	71	0

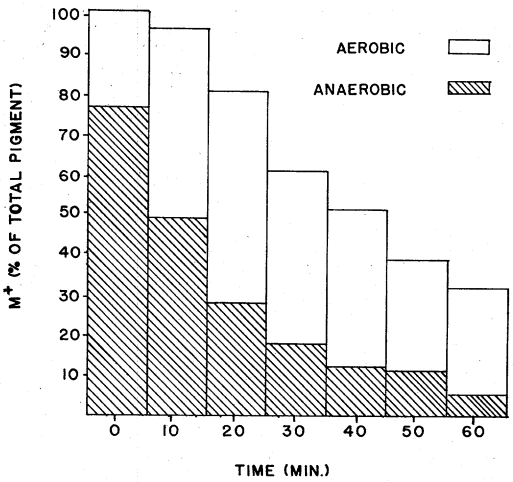

Fig. 7. Comparison of M^+ reduction in ground beef containing 40 mg% DPN under aerobic vs. anaerobic conditions.

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Abbreviations used: ATP = adenosine triphosphate, DPN = diphosphopyridine nucleotide (also known as niacin adenine dinucleotide), DPN^+ and DPNH = oxidized and reduced states, respectively, of DPN; ETC = electron-transport chain; FD = flavoprotein DPN dehydrogenase; FS = flavoprotein succinic dehydrogenase; LD = lactic dedrogenase; M = reduced myoglobin; M^+ = metmyoglobin; MO_2 = oxymyoglobin; MRA = metmyoglobin-reducing activity; PO_2 = oxygen tension; ppm = parts per million.

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